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Mutagen Formation in a Model Beef Supernatant Fraction. IV. Properties of the System

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To identify the precursors and elucidate the reaction conditions that yield heterocyclic amine mutagens in cooked meat products and fish, we have used a supernatant 2 (S_2) fraction prepared from H_2O-homogenized lean round steak. Compounds (MW <500) in S_2 are the sources of the microsomal-dependent, Salmonella TA 1538 mutagenic activity in open boiled (aqueous), 200°C pressure-heated (aqueous), or 200 to 300°C oven-baked (freeze-dried) homogenates. Combined incorporation-HPLC experiments show that they are also the precursors for frameshift mutagen formation in the outer surfaces of 200°C griddle-fried ground beef. Maximal stimulations of boiled S_2 mutagenic activity are given by 10 mM Trp, 2.5 mM creatine phosphate (CP), and synergistically by 10 mM Trp + 2.5 mM CP + 1.0 mM $FeSO_4$ (a mixture abbreviated as S_2^*). Boiling S_2 for 30 hr at the acidic optimum pH of 4.0 → 600 TA 1538 revertants (no additions) and 1,400 revertants (+CP), while S_2^* → 24,000 revertants/10^8 bacteria/g of dry beef. By the criteria of HPLC, paper electrophoresis, and resistance of the active HPLC fractions to acid-nitrite inactivation, boiled S_2 contains 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), 3-amino-1-methyl-5H-pyrido[4,3-*b*]indole (Trp-P-2) and a minor amount of 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ). Boiling S_2 with CP doubles the IQ, halves the Trp-P-2, produced traces of MeIQ, and generates an unknown nitrite-resistant mutagen. Boiled S_2^* contains these same four mutagens, but both the IQ and Trp-P-2 are increased and large amounts of Trp-P-1 also are generated. The identities of IQ, Trp-P-2, and Trp-P-1 were verified by purification and by light-absorption and mass spectra. Their increments in stimulated S_2 indicate that Trp (or its degradation products) and CP (or its degradation products) are the beef juice precursors for the indole ring in Trp-P type mutagens and the NH_2-imidazole ring in IQ-type mutagens, respectively. Aqueous (pressure) heating or oven-baking S_2 for 2 hr at 200°C greatly elevates its TA 1538 activity → 45,000 revertants/10^8 bacteria/g of dry beef; dry heating at 300°C → ~180,000 revertants/g of dry beef. Along with the increases in total TA 1538 activity at 200 to 300°C, the number of mutagens formed from the <500 MW S_2 precursors also multiplies. IQ is one of the many mutagens generated by dry heating S_2 at 200°C. Thus, once the soluble S_2 precursors have been concentrated to a freeze-dried powder, aqueous conditions are not essential to obtain heterocyclic amine mutagens at this ordinary cooking temperature. Water behaves as an important reactant that influences the relative proportions of HPLC polar, nitrite-resistant, IQ-type as opposed to HPLC nonpolar, nitrite-sensitive, non-IQ-type mutagens. Dry heating S_2 favors the former.

Introduction

Because of its sensitivity to most tumor initiators, the Ames/Salmonella assay has been successfully employed to detect the presence of microsomal (S-9)-dependent frameshift mutagens in a variety of cooked nitrogen-rich foods, especially meats and fish (*1–4*). Thus far, nine heterocyclic amine mutagens have been isolated and identified structurally from 500 to 600°C pyrolyzates of amino acids and proteins, while three have been purified and identified structurally from broiled fish or fried ground beef (*4–9*). To date, seven of these heterocyclic amines have proven to be carcinogenic in rodent feeding studies (*10–15*). Among these seven mutagenic carcinogens are IQ, which occurs in broiled sardines and broiled or fried beef, Trp-P-2, which is found in broiled sardines, and Trp-P-1, which is reportedly in both broiled sardines and broiled beef (*6–9*). Most of the heterocyclic amine mutagens that have been identified from cooked fish or beef and various pyrolyzates (exceptions IQ, MeIQ, and MeIQx) are inactivated by deamination with nitrite under acidic conditions (*7,8*). Thus, the percentage of S-9 requiring frameshift mutagenic activity that is resistant to acid-nitrite provides an estimate of the fraction of IQ-type (i.e., NH_2-imida-

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zole containing) versus non-IQ-type (e.g., Trp-P-2, Trp-P-1) activity in a crude sample.

In order to devise ways to inhibit the thermogenesis of potent mutagens-carcinogens during the cooking or heat-processing of foodstuffs, it is necessary to know the key precursor sources, heat conditions, and reaction sequences that contribute to their appearance. Consequently, a large number of model systems have been devised by aqueously or dry heating together arbitrary concentrations or amounts of compounds that are known to exist in proteinaceous foods (*16–21*). Binary and ternary mixtures of sugars, NH_4OH, amino acids, proteins, fatty acids, vitamins, and creatine (creatinine) produce Salmonella mutagenic activity when heated at 100 to 250°C, but in most cases the mutagenic products remain to be identified and quantified. The foregoing is quite different from the modeling approach that we have taken, namely study mutagen formation under a variety of heating conditions with a fraction derived directly from beef muscle (*20*). While our model system has not yet been simplified to a minimal number of essential reactants, it is unique and potentially offers two significant advantages: (1) the naturally occurring mutagen precursors are present near their levels and in the same relative quantities as in raw meat muscle and (2) a priori it should be possible to generate many of the heterocyclic amine mutagens that arise in cooked meat products and fish. We were guided toward the use of an H_2O-soluble beef fraction, in part, because various forms of beef, collectively, are a large source of the daily protein in the U.S. diet (*22*). In addition, Commoner et al. found that Difco beef extract and fried hamburger contain some similar mutagens that form at moderate temperatures (*23*). This has since been confirmed by the isolation of IQ and MeIQx from both bacterial and food grade beef extracts and from fried or broiled beef (*7–9, 24–27*). In this paper, we define the mutagen producing characteristics of a soluble beef supernatant fraction, describe its stimulation by select agents present in beef juice, and demonstrate that <500 MW compounds are the mutagen precursors, irrespective of the heating method used. This includes frying within a ground beef matrix. Evidence, during boiling, for the formation of IQ, Trp-P-2, Trp-P-1, traces of MeIQ, and an unknown mutagen is given. However, the same beef fraction, when dry-heated, is a rich source of predominantly IQ-type mutagenic activity.

Materials and Methods

Unlabeled IQ, unlabeled MeIQ, and [5-^{3}H]IQ (78,000 cpm/ng) were synthesized by Waterhouse and Rapoport (*28*). Unlabeled Trp-P-2 was purchased from Wako Chemicals, Inc.; [G-^{3}H]Trp-P-2 (96,000 cpm/ng) was tritiated by Moravek Biochemicals, Inc. Unlabeled Trp-P-1 and MeIQx were kindly supplied by Drs. T. Sugimura and S. Sato, National Cancer Center Research Institute, Tokyo, Japan. All other chemicals and extraction solvents were obtained from commercial sources (Calbiochem-Behring Corp., Sigma Chemical Co., Aldrich Chemical Co., or Mallinckrodt Chemical Co.).

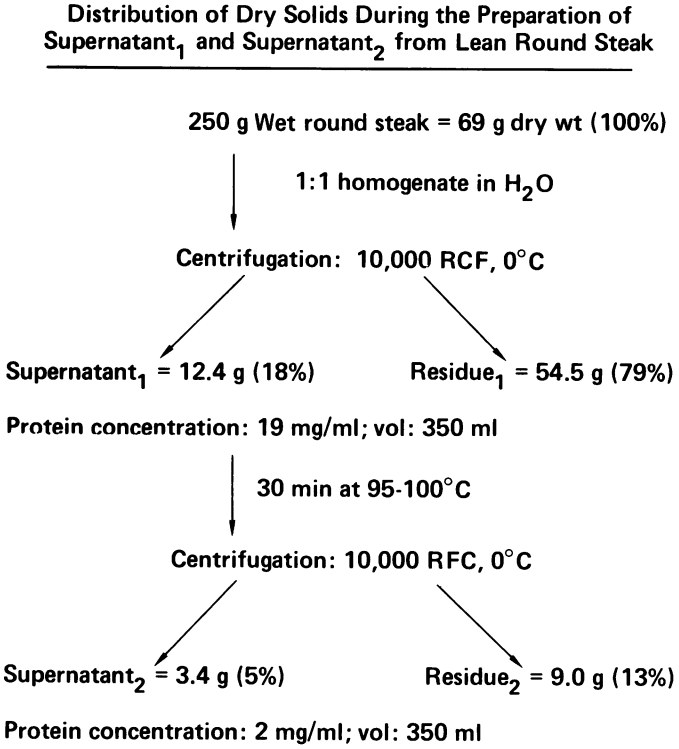

FIGURE 1. Preparation of fractions from round steak.

The beef fractions, supernatant 1 (S_1), supernatant 2 (S_2), residue 1 (R_1), and residue 2 (R_2), were prepared from trimmed choice-grade round steak (72.4% H_2O; 11% fat) (Fig. 1) (*20*). Subfractionation of S_2 was performed by molecular ultrafiltration through Diaflo membranes (Amicon Corp.) (*20*). Boiling at a constant volume (300 mL/60 g dry beef) was carried out routinely at pH 4.0 for 14 hr (stimulation experiments) or 30 hr (mutagen characterization and isolation experiments) in mechanically stirred, Teflon-covered beakers that were heated with an oil bath (*20*). Aqueous (pressure) heating (100 mL/20 g dry beef) at 200°C was facilitated by the use of a Teflon-lined stainless-steel Parr bomb (120 mL capacity, Parr Instrument Co.) that was placed in an oven (Thermolyne ash furnace). Extensively freeze-dried beef fractions were dry-heated in foil-covered Pyrex lyophilization flasks placed in the oven. All dry-heated fractions were resuspended or redissolved into distilled H_2O (5 mL/g original dry beef equivalent) prior to extracting their mutagenic activity. Boiled, solution (pressure) heated, or dry-heated materials were adjusted to pH 12.0 with NaOH and extracted six times with an equal volume of methylene chloride + methanol (3:1) (*20*). Centrifugation was used to disrupt any emulsions and to separate the liquid phases cleanly. The pooled organic phases were taken to dryness under vac-

uum by rotary evaporation. The residues were then dissolved in dimethyl sulfoxide, methanol, or H_2O, depending on whether their intended use was mutation assays only, HPLC/electrophoretic analyses, or scaled-up mutagen purifications, respectively (*20,29*). Pairs of ground, round steak patties (100 g, 9 cm in diameter × 1.5 cm in thickness) that had been uniformly kneaded with known equivalents of S_2, R_1, or R_2 (Fig. 1) were fried 10 min/side at 200°C (*30*). The outer 3-mm surface layers were removed, homogenized with a Polytron (Brinkmann Instruments) in three volumes of methanol, and then filtered by vacuum suction through Whatman No. 1 paper, after magnetically stirring the homogenate 20 to 30 min at room temperature to promote equilibration. The filtered solids were extracted five more times in the same manner. Then the combined methanolic filtrates were subjected to rotary evaporation. The methanolic residues were taken up in distilled H_2O (5 mL/g of original uncooked patty wt) and pH 12.0 organic extracts were prepared as described above and in Ref. 20.

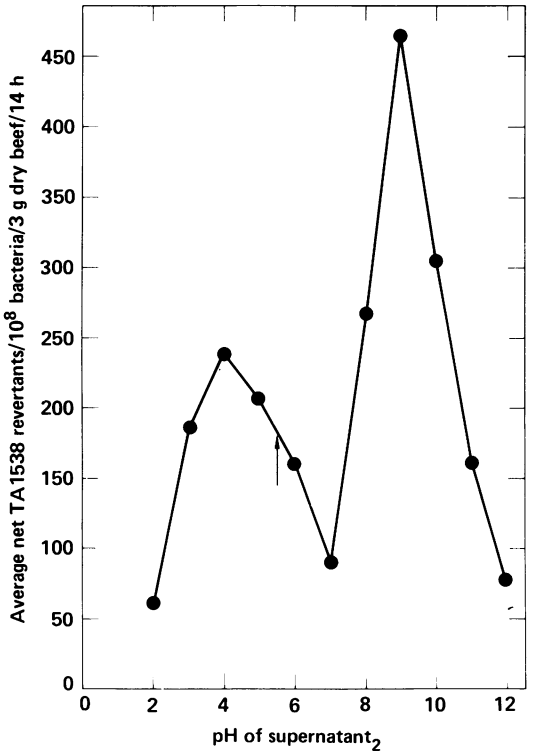

FIGURE 2. pH Dependence of mutagen formation from supernatant 2. 300-mL volumes of supernatant 2 were boiled for 14 hr at the indicated pH. The arrow notes the natural pH of the supernatant 2 fraction. It is typically 5.5–5.8 (*20*).

Ames/Salmonella TA 1538 and TA 100 mutation assays were performed by the standard pour-plate method (*31*), routinely using 2.0 mg of Aroclor 1254-induced rat liver S-9 per plate (*20,27*). All tabulated bacterial mutation results were derived from linear four-level dose-response curves (*20*). Resistance to acid-nitrite inactivation was determined by a slightly modified, scaled-down version of the procedures of Yoshida et al. (*32*) and Tsuda et al. (*33*). Duplicate aliquots of the samples and standards capable of inducing 5–10 × 10^4 TA 1538 revertants were incubated for 30 min at 37°C in 0.1 M HCl ± 20 mM $NaNO_2$ in a volume of 1.0 mL. The incuabion mixtures were then neutralized with 150 μL of 1 M $NaHCO_3$ and passed through 0.45 μm filters. Each filtrate was assayed for the remaining S-9 dependent TA 1538 activity and the appearance of any new S-9 independent activity. None of the latter was observed.

A Hewlett-Packard 1084B liquid chromatograph,

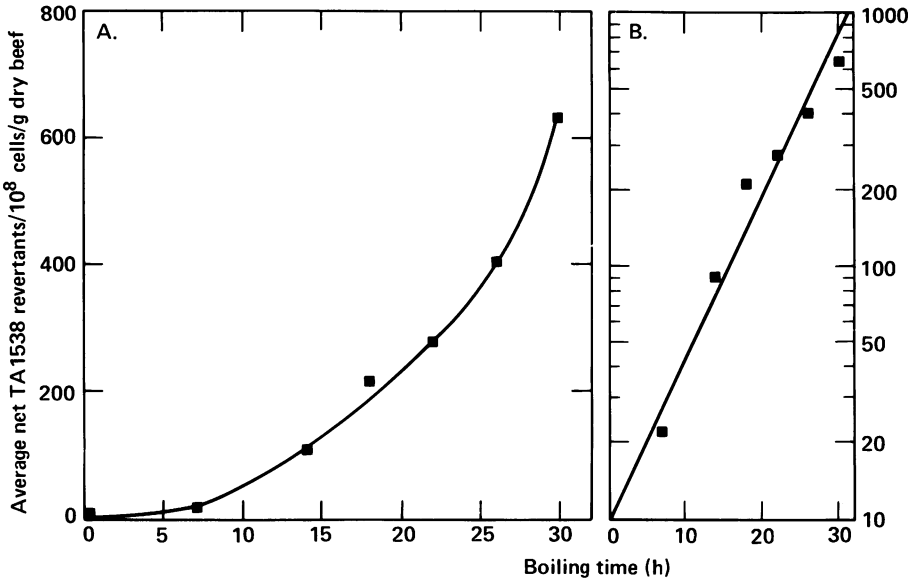

FIGURE 3. Time course of mutagen formation from supernatant 2 at pH 4.0.

equipped with an HP 1040A detector and an Altex 210 sample injector, was used in all of the HPLC work. In the large-scale mutagen isolations, the preparative and semimicro reverse-phase HPLC steps were carried out as in previous studies (*20,27*). Analytical reverse-phase HPLC on a 4.6 × 250 mm Partisil ODS-3 C_{18} silica (5 μm particles) column (Whatman) was employed to compare the mutagen elution profiles of beef S_2 heated in several different ways. Eluate fractions (0.6 mL) were collected at a flow rate of 1.2 mL/min. Elution conditions were: 40% methanol in H_2O → 50% methanol in H_2O (0–20 min) → 70% methanol in H_2O (20–30 min) → 100% methanol (30–32 min) → 100% methanol (32–40 min). Heptane sulfonate (5 mM; pH 5.5) was included in the elution solvents as a counter ion. Whatman 3MM sheets (30 cm × 30 cm) and strips (6 cm × 30 cm) were used for preparative ascending paper chromatography and preparative electrophoresis (10% formic acid, pH 1.5, 2 hr, 400 V, Beckman Model R chamber), respectively (*29*). Narrower strips (3 cm × 30 cm) and thinner paper (Whatman No. 1) were used for the electrophoresis of active analytical reverse phase HPLC peaks. Subsequently, the TA 1538 activity was dissolved from 3 cm × 0.5 cm segments of these No. 1 strips by cutting each segment into small pieces and soaking them in 0.3 to 0.5 mL of dimethyl sulfoxide for several hours. Low resolution probe mass spectra were obtained with a Hewlett-Packard 5282A spectrometer operating at 70 eV and a source temperature of 200°C.

Results

Influence of the Boiling Conditions and the Molecular Fractionation of Supernatant 2

Although S_2 represents only 5% of the beef dry weight and 10% of the H_2O-soluble protein (Fig. 1), it is the source of all the S-9 dependent TA 1538 activity that can be generated by a prolonged boiling of the entire homogenate (*20*). Activity from S_2 is formed optimally at pH 4.0 and 9.0 (Fig. 2) and increases exponentially with the boiling time (Fig. 3). No mutagenic activity can be detected upon the conversion of S_1 to S_2. Following our initial report of the pH-dependence of mutagen formation in beef S_2 (*34*), a pH-activity pattern very similar to that in Figure 2 was also observed for a boiled pork juice concentrate (*35*). By our procedures, boiling pork S_2 for 14 hr at pH 4.0 yields 70% as much TA 1538 activity as boiling beef S_2 (*21*). We have chosen

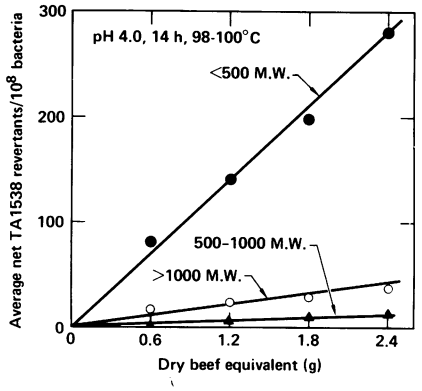

FIGURE 4. Source of the mutagenic activity when supernatant 2 is subfractionated by molecular ultrafiltration.

Table 1. Effects of single amino acids and combinations of amino acids on the mutagenicity of boiled round steak supernatant 2.

L-Amino acid addition(s) (2.5 mM each)	Avg. net TA 1538 revertants/10^8 bacteria/g dry beef relative to supernatant 2 alone[a]	
	pH 4.0[b]	pH 9.0[b]
Lys + His + Arg	1.7	1.3
His	1.3	1.2
Thr + Ser	1.0	1.7
Asp + Asn + Glu + Gln	1.3	1.0
Pro	2.5	1.1
Gly	1.6	1.1
Met	1.6	1.1
Ala + Val + Ile + Leu	1.1	0.9
Cys	4–5[c]	0.8
Tyr + Phe + Trp	10–14	1.8
Trp	10–14[c]	2.0
Tyr + Phe	0.9	0.8

[a] Values for supernatant 2 alone ranged from 85 to 105 revertants/g dry beef at pH 4.0.
[b] Boiling was at 98–100°C for 14 hr at the indicated pH.
[c] Relative values for Cys or Trp boiled alone are 0.1–0.3.

Table 2. Effects of various nonamino acid nitrogenous compounds on the mutagenicity of round steak supernatant 2.[a]

Addition (2.5 mM each)	Avg net TA 1538 revertants/10^8 bacteria/g dry beef
None	90
Creatine	107
Creatinine	107
Creatine phosphate	600
Niacin (nicotinic acid)	182
Adenine + guanine + hypoxanthine + xanthine	152
ATP	107
GTP	79
Sarcosine	79
N,N-Dimethylglycine	60
1-Methyl-L-histidine	58
L-Carnosine	68
L-Anserine	65
Taurine	65
Pyrazine	97
2-Methylpyrazine	90
2,5-Dimethylpyrazine	98
2-Pyrazinecarboxylic acid	96
2,3-Pyrazinedicarboxylic acid	94
2-Methylpyrazine + creatine phosphate	483
2,5-Dimethylpyrazine + creatine phosphate	496
D-Ribose + creatine phosphate	555
D-Glucose + creatine phosphate	540

[a] Boiling was at 98–100°C for 14 hr at pH 4.0.

Table 3. Synergistic interaction between L-tryptophan, creatine phosphate, and a metal ion in the stimulation of mutagen formation from round steak supernatant 2.[a]

Addition(s) or deletions[b]	Avg net TA 1538 revertants/ 10^8 bacteria/g dry beef[c]			Avg net TA 100 revertants/ 10^8 bacteria/g dry beef	
L-Trp + CP + $FeSO_4$	5500	(0)		610	
L-Trp + CP	3040	(0)		615	
L-Trp + $FeSO_4$	3240	(8)		730	
CP + $FeSO_4$	534	(9)		102	
L-Trp + CP + $FeSO_4$ minus supernatant 2	58			145	
None	92	(4)		25	
L-Trp	1780	(0)		393	593
CP	460	(4)	2467	100	
$FeSO_4$	135	(2)		75	
D-glucose	143	(3)		80	
L-Trp + CP + $FeSO_4$ + D-glucose	3400	(0)		570	

[a] Boiling was at 98–100°C for 14 hr at pH 4.0.

[b] Where indicated, L-tryptophan (L-Trp), creatine phosphate (CP), $FeSO_4$, and D-glucose were added at concentrations of 10 mM, 2.5 mM, 0.5 mM, and 10 mM, respectively.

[c] Numbers in parentheses give the average net TA 1538 revertants/10^8 bacteria/g dry beef when the Salmonella mutagen assays lacked S-9.

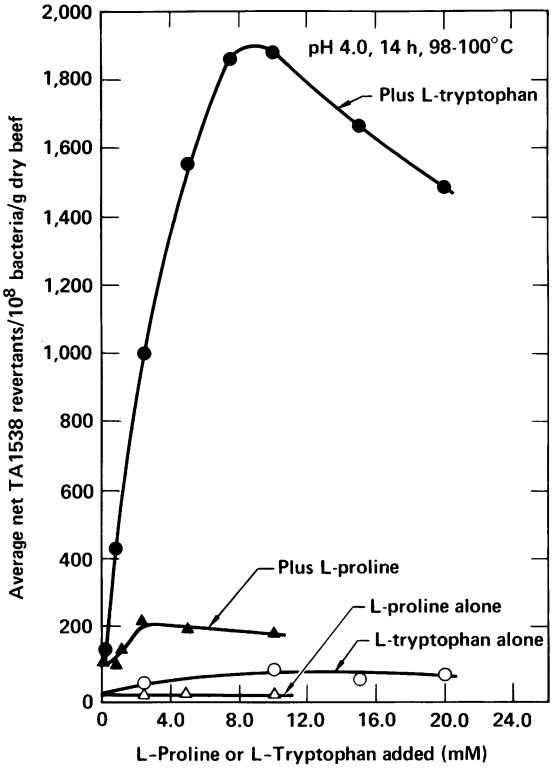

FIGURE 5. Effects of the L-tryptophan and L-proline concentrations on the mutagenicity of boiled supernatant 2.

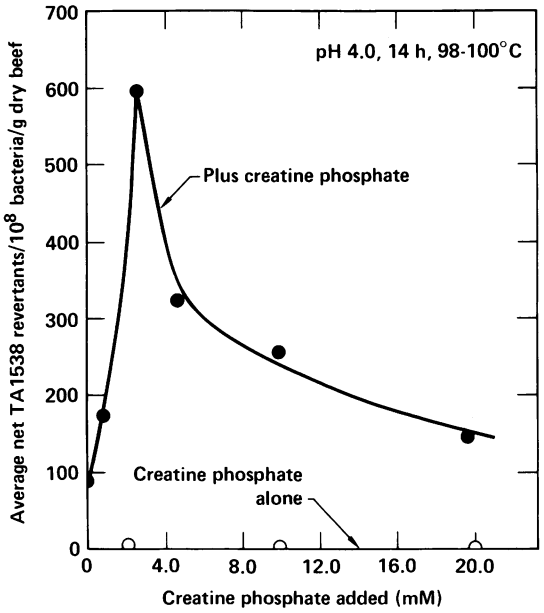

FIGURE 6. Effects of the creatine phosphate concentration on the mutagenicity of boiled supernatant 2.

to boil routinely at the acidic pH optimum (4.0) because it is closer to the pH (5.6) of round steak homogenate and ground beef muscle. Figure 4 shows that most of the frameshift activity in boiled S_2 arises from precursors that have molecular weights of <500 daltons.

Stimulation of Boiled Supernatant 2

Proteolysis experiments and amino acid analyses suggested that select amino acids in combination with other low molecular weight compounds are the precursors for the mutagens in boiled beef muscle (*21*). Stimulation studies were therefore carried out (*36*) by boiling S_2 with increased levels of the amino acids it contains (*21*) and with various other compounds that are found in meat juices (*37*). In these experiments, the test compound(s) was(were) added to S_1 (Fig. 1) before it was converted to S_2 for 14 hr or 30 hr of extensive boiling. Among the 20 common amino acids, only Trp, Cys, and Pro increase the mutagenic activity of pH 4.0 boiled S_2 more than 2-fold (Table 1). Maximal enhancements of boiled S_2 are given by 10 mM Trp (Fig. 5), 2.5 mM Pro (Fig. 5), and 10 mM Cys (6- to 7-fold, not shown). Stimulation by Pro is minor relative to that by Trp (Fig. 5). The enhancement by Cys has not been studied in detail

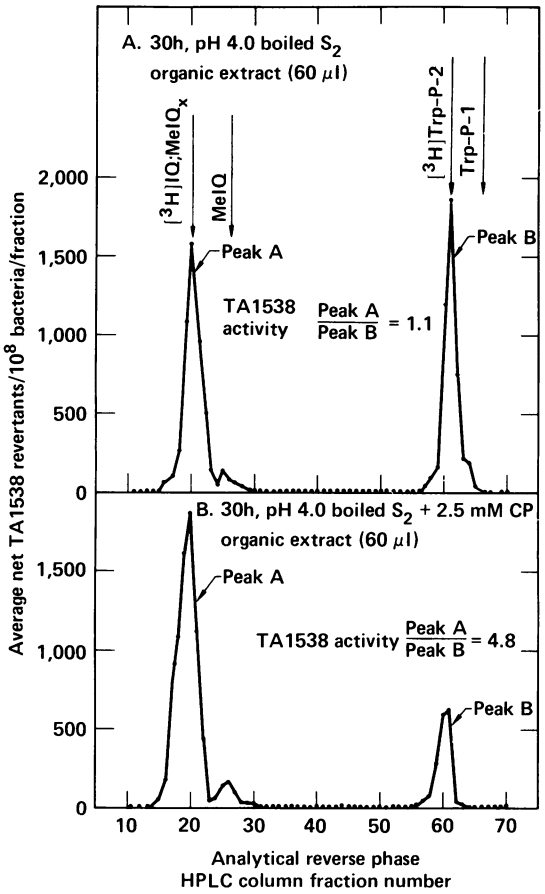

FIGURE 7. Analytical reverse phase HPLC of the alkaline organic extracts from supernatant 2 boiled alone (*A*) and boiled with 2.5 mM creatine phosphate (*B*). The vertical arrows denote the coelution maxima of [^{3}H]IQ, MeIQx, [^{3}H]Trp-P-2, and Trp-P-1 when individually added to the sample extracts and chromatographed. In part (*A*), 60 μL of a 250 μL total extract sample and in part (*B*), 60 μL of a 500 μL total extract sample were applied to the column.

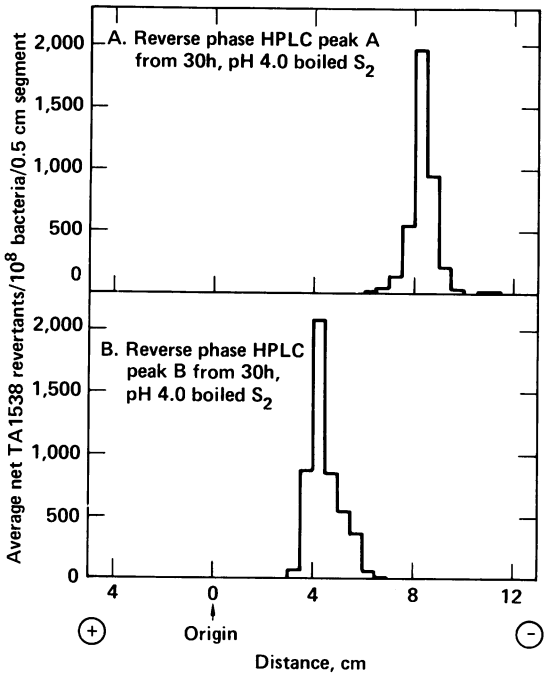

FIGURE 8. Paper strip electrophoresis (Whatman No. 1) of analytical reverse phase HPLC peaks A and B from boiled supernatant 2.

because it is also a reducing agent and it lowers the activity of boiled S_2 supplemented with Trp ± Pro. Meat juices also contain a number of nonamino acid nitrogeneous compounds, along with the sugars glucose > fructose > ribose (*37*). Moreover, pyrazines are major Maillard reaction intermediates that form rapidly in browning reaction model systems and in all types of cooked meats (*16,38*). Among 21 nitrogenous compounds, only creatine phosphate (CP) markedly enhanced the mutagenic activity of boiled S_2 (Table 2). None of 11 sugars (including glucose, fructose, and ribose) augmented its activity when added at concentrations of 2.5 mM or 10 mM (*36*). The endogenous concentrations of glucose and ribose in S_1 are approximately 1 to 3 mM. Also, glucose, ribose, and two common pyrazines do not further enhance the stimulation produced by CP (Table 2). CP stimulation is maximal at 2.5 mM and occurs within a narrow concentration

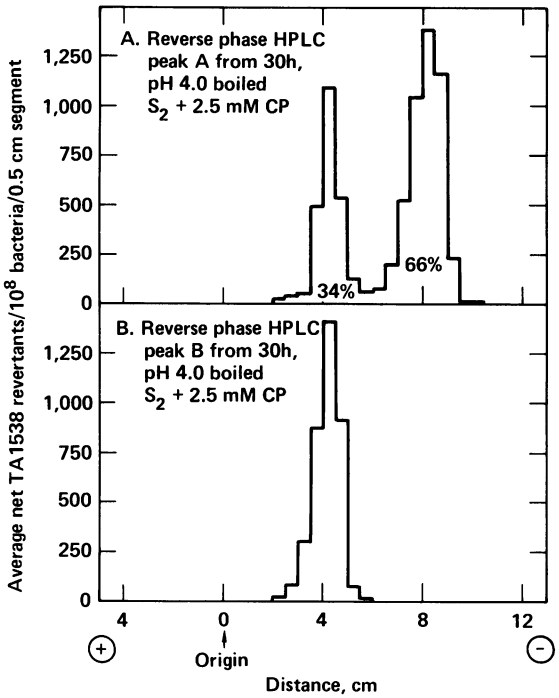

FIGURE 9. Paper strip electrophoresis (Whatman No. 1) of analytical reverse phase HPLC peaks A and B from supernatant 2 boiled with creatine phosphate.

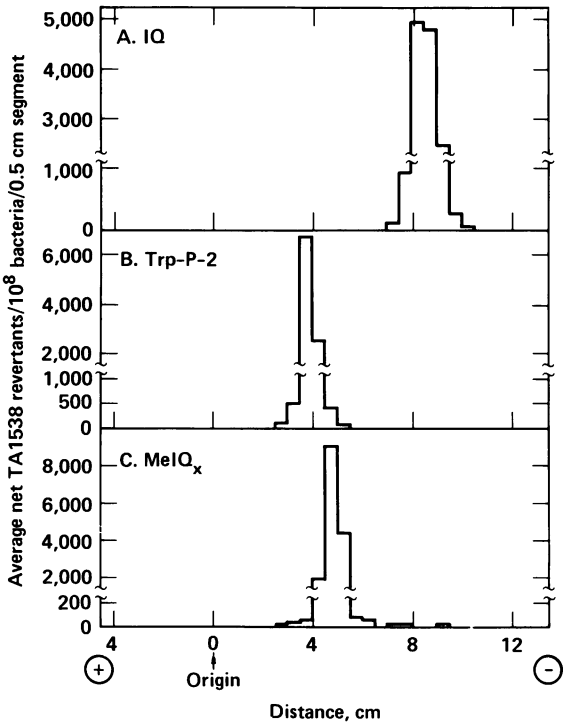

FIGURE 10. Paper strip electrophoresis (Whatman No. 1) of IQ(A), Trp-P-2 (or Trp-P-1 not shown) (B), and MeIQx (C).

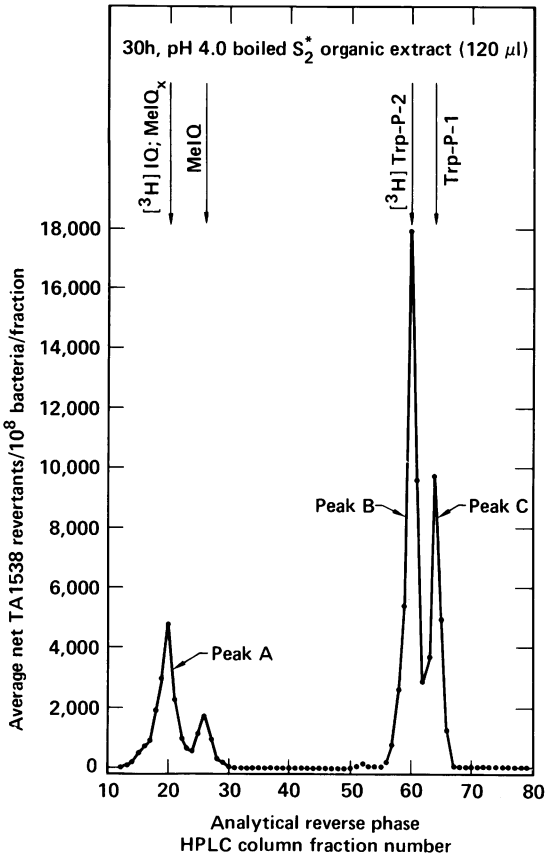

FIGURE 11. Analytical reverse phase HPLC of the alkaline organic extract from boiled supernatant 2*. 120 µL of a 1.0 mL total extract sample were applied to the column.

range (Fig. 6). Boiling S_2 in the presence of various trace metal ions does not enhance boiled S_2 TA 1538 activity. However, several metal salts (e.g., $FeSO_4$, $CoCl_2$, and $NiCl_2$), in combination with Trp and CP, stimulate its frameshift mutagenic activity by 50- to 55-fold (Table 3). Glucose partially inhibits the synergistic increase in the TA 1538 activity, but not the much smaller additive increase in TA 100 activity. We will routinely refer to the synergistically stimulated mixture as boiled supernatant 2* (S_2^*). Endogenous heat-labile, <500 MW compounds in beef S_1 are essential co-reactants (co-precursors) in this synergistic stimulation (R. T. Taylor and E. Fultz, results to be published).

Characterization and Identification of the Mutagens Formed in Supernatant 2 Boiled ± Creatine Phosphate and in Boiled Supernatant 2*

We first determined the number and types of frameshift mutagens that form in 30 hr boiled S_2, S_2 + 2.5 mM CP, and S_2 + 10 mM Trp + 2.5 mM CP + 1.0 mM $FeSO_4$ (S_2^*). By the criteria of analytical reverse-phase HPLC (Fig. 7*A*) and paper strip electrophoresis (Figs. 8 and 10), boiled S_2 appears to contain IQ and Trp-P-2, plus a trace of MeIQ. The mutagenic HPLC peaks A and B (activity ratio = 1.1), correspondingly, co-elute with [^{3}H]IQ and [^{3}H]Trp-P-2 and are nitrite-resistant

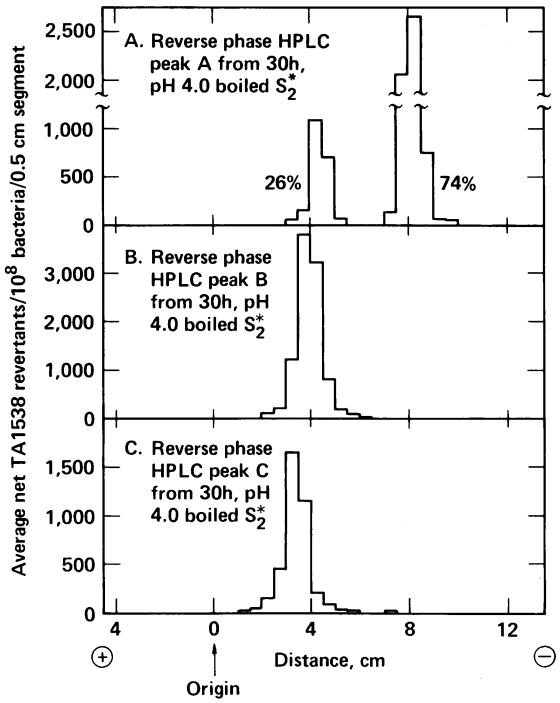

FIGURE 12. Paper strip electrophoresis (Whatman No. 1) of analytical reverse phase HPLC peaks A, B, and C, from boiled supernatant 2*.

Table 4. Purification of the mutagens from 30 hr, pH 4.0 boiled beef supernatant 2 (peaks A and B) and supernatant 2* (Peak C).[a]

Step or fraction	Overall % TA 1538 activity recovery	Overall fold TA 1538 activity enrichment[b]
First alkaline (pH 12.0) extraction with methylene chloride:methanol (3:1)	100	1.0
Second alkaline extraction after a pH 2.0 extraction with methylene chloride	96	6.3
Preparative reverse phase HPLC		
Peak A	44	28
Peak B	30	66
Peak C	10	17.6
Ascending paper chromatography H_2O:nBuOH:iPrOH:HAc (100:100:70:1)		
Peak A	33	485
Peak B	29	157
Peak C	9	17.7
Ascending paper chromatography nBuOH:HAc:H_2O (12:3:5)		
Peak A	30	2658
Peak B	24	339
Peak C	8	24.2
Paper strip (6 × 30 cm) electrophoresis (10% formic acid, pH 1.5)		
Peak A	18	3616
Peak B	15	1562
Peak C	6	21.8
Semimicro reverse phase HPLC		
Peak A	12	4758
Peak B	12	2366
Peak C	4.3	16.5

[a] Mutagen peaks A and B were purified from 960 g dry beef equivalents of beef supernatant 2 (5.31×10^5 total initial TA 1538 revertants of activity), while mutagen peak C was isolated from 480 g dry beef equivalents of beef supernatant 2* (9.81×10^6 total initial TA 1538 revertants of activity).

[b] Increase in mutagenic specific activity is based on the TA 1538 revertants per A_{260nm} unit.

and nitrite-sensitive, respectively. Boiled S_2 + CP contains these same mutagens (Figs. 7*B*, 9, and 10), plus an unknown nitrite-resistant mutagen. It elutes as part of HPLC peak A (Fig. 7*B*), but migrates electrophoretically like MeIQx (Figs. 9*A* and 10). However, analytical normal phase HPLC (not shown) substantiated that this polar mutagen, which constitutes one-third of the boiled S_2 + CP peak A activity (Fig. 9*A*), is not MeIQx. Boiled S_2 + CP, therefore, contains peak A, unknown mutagen, and peak B in the activity ratios of 3.2:1.6:1.0. Reverse phase HPLC indicates that boiled S_2^* also contains IQ, Trp-P-2, and a minor amount of MeIQ (Fig. 11). In addition, another mutagen (peak C) elutes at the same position as Trp-P-1. Paper electrophoresis (Figs. 10 and 12) and acid-nitrite resistance assays are consistent with the presence of IQ plus the unknown mutagen in HPLC peak A, Trp-P-2 in peak B, and Trp-P-1 in peak C.

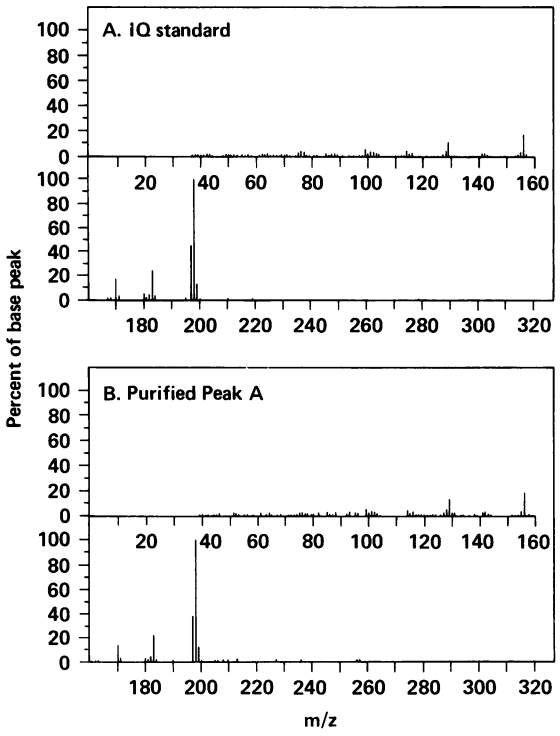

FIGURE 13. Mass spectra of IQ (0.5 μg) (*A*) and purified HPLC peak A (*B*).

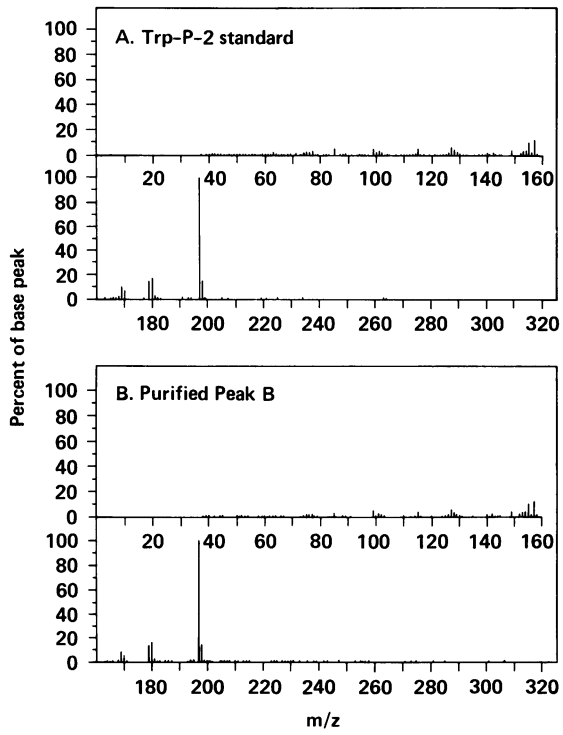

FIGURE 14. Mass spectra of Trp-P-2 (0.5 μg) (*A*) and purified HPLC peak B (*B*).

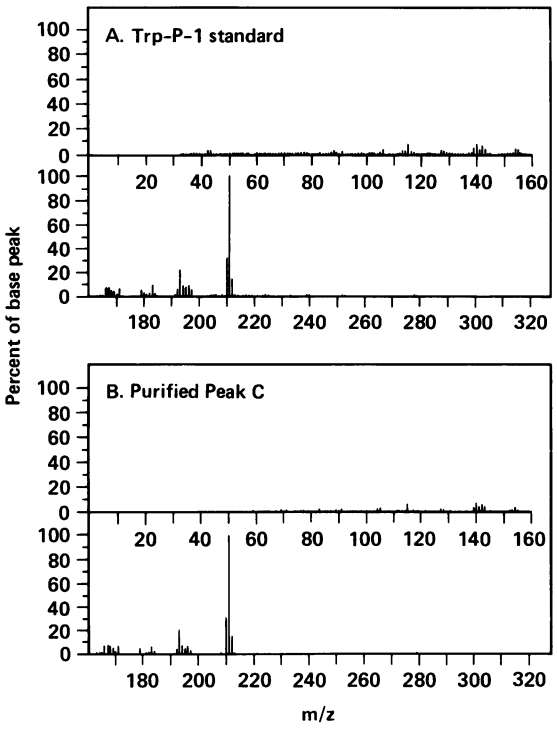

FIGURE 15. Mass spectra of Trp-P-1 (1.0 μg) (*A*) and purified peak C (*B*).

Paper electrophoresis at pH 1.5 (Fig. 10) is a very simple and useful technique for detecting the presence of IQ and MeIQ in a mutagenic organic extract or HPLC fraction. Because of their doubly positive net charges (2 protonations), the electrophoretic mobilities of IQ and MeIQ are about twice those of the other reported food-related, heterocyclic amine mutagens. If one finds any TA 1538 activity that migrates as a fast moving electrophoretic band, and after elution from the paper it requires S-9 and is acid-nitrite resistant, it is almost certain to be IQ or MeIQ. By increasing either the run time or the voltage, one can then distinguish MeIQ from IQ because the latter moves slightly faster.

To verify the identities of the foregoing HPLC peaks A, B, and C, large-scale purifications were undertaken. Mutagenic peaks A and B were purified to homogeneity 4800-fold and 2400-fold, respectively, and stockpiled from multiple 960 g equivalent batches of dry beef (Table 4). By the same isolation steps, HPLC peak C was purified to homogeneity after only a 17-fold enrichment, based on TA 1538 activity/A_{260nm} unit. Ample amounts of mutagenic peak C were isolated from a single 480 g equivalent batch of dry beef (Table 4). Since peak C represents only 10 to 15% of the total activity in boiled S_2^* (Fig. 11), its true corrected recovery in Table 4 is near 40% rather than the apparent 4.3%. The light-absorption spectra of purified peaks A, B, and C exactly match those of authenic IQ, Trp-P-2, and Trp-P-1, respectively (*29*), and their mass fragmentation spectra closely mimic those of the same corresponding standards (Figs. 13–15). Table 5 summarizes the mutagenic activities, the acid-nitrite resistances, and the amounts of the major mutagens in 30 hr boiled S_2, S_2 + CP, and S_2^*. Boiled basal S_2 contains IQ, Trp-P-2, and traces of MeIQ (Fig. 7*A*). In the presence of added CP, minor amounts of MeIQ again appear (Fig. 7*B* plus paper electrophoresis data not shown), but more IQ (2-fold) and an unknown nitrite-resistant mutagen form (Fig. 9*A*) at the expense of Trp-P-2 (Table 5). Boiled S_2^* contains more MeIQ (Fig. 11 and paper electrophoresis data not shown) and the same unknown nitrite-resistant mutagen (Fig. 11*A*). However, the synergistic stimulation (Table 3) markedly increases both IQ (8-fold) and Trp-P-2 (34-fold) and generates large amounts of Trp-P-1 (Table 5). Together, Figure 11 and Table 5 are a good illustration of the fact that Salmonella activity does not equate to quantity of mutagen in mixtures that contain mutagens of widely differing mutagenic potencies. In our hands, the TA 1538 mutagenicities of purified peak A (or IQ), purified peak B (or Trp-P-2), and purified peak C (or Trp-P-1) per A_{260nm} unit are 1.12×10^6, 5.56×10^6, and 3.0×10^4, respectively. These results demonstrate that cooked food-related heterocyclic amine mutagens (*4–9*) can form in H_2O at 100°C, either from small precursors already in the S_2 fraction or when stimulated further by select compounds that appear to be rate limiting in S_2.

Table 5. Summary of the amounts of IQ, Trp-P-2, and Trp-P-1 in boiled supernatant 2 ± CP and supernatant 2.*

Boiling mixture, 30 hr, pH 4.0	Avg net TA 1538 revertants/10^8 bacteria/g dry beef[a,b]	TA 1538 30 min 37°C acid-nitrite resistance[c]	Mutagen, ng/g dry beef equivalent[b]		
			IQ	Trp-P-2	Trp-P-1
Basal beef supernatant 2	517–640	47%	1.4	3.2	ND[d]
Basal beef supernatant 2 + 2.5 mM CP	1,240–1,510	81%	2.9	1.9	ND[d]
Supernatant 2* (Supernatant 2 + 10 mM L-Trp + 2.5 mM CP + 1.0 mM $FeSO_4$)	20,438–27,400	13%	11.1	110.0	875

[a] Determined with 2.0 mg of S-9 protein.

[b] To convert mutagen yields to ng/g wt wet equivalent of original beef each value should be multiplied by 0.28. To convert mutagen yields to ng/g of supernatant 2 dry weight each value should be multiplied by 20.

[c] Percent of the acid control lacking $NaNO_2$. When $NaNO_2$ was omitted from the incubation mixture, the yields of mutagenic activity were >95% of the original sample TA 1538 activities. All residual activities were S-9 dependent. Corresponding data for the standards IQ, Trp-P-2, and Trp-P-1 were 95%, 0%, and 1%, respectively.

[d] Not detectable.

Table 6. Source of the mutagenic activity when beef round steak fractions are heated at frying and baking temperatures.

Fraction	Heating Conditions	Avg net TA 1538 rev./10^8 bacteria/g dry beef[b]	Avg net TA 100 rev./10^8 bacteria/g dry beef[b]	TA 1538 30 min 37°C acid-nitrite resistance, %[c]
Whole homogenate	Solution (pressure)	38,000 (0)	3,575 (0)	63%
Residue 1	2 hr, pH 4.0, 200°C	500 (0)	0 (0)	ND[d]
Supernatant 1		43,700 (0)	3,825 (0)	65%
Supernatant 2		42,000 (0)	3,670 (0)	58%
<500 MW cut of supernatant 2		38,800 (0)	3,640 (0)	61%
Whole homogenate	Oven-baked	27,200 (0)	2,550 (0)	91%
Residue 1	2 hr, 200°C[a]	72 (0)	0 (0)	ND[d]
Supernatant 1		45,200 (0)	5,450 (0)	86%
Supernatant 2		44,000 (0)	4,850 (0)	94%
<500 MW cut of supernatant 2		45,500 (0)	5,500 (0)	89%
Whole homogenate	Oven-baked	128,000 (0)	12,400 (0)	92%
Residue 1	2 hr, 300°C[a]	3,320 (0)	562 (0)	ND[d]
Supernatant 1		186,400 (0)	20,000 (0)	86%
Supernatant 2		190,000 (0)	18,600 (0)	92%
<500 MW cut of supernatant 2		188,000 (0)	17,600 (0)	90%

[a] Each corresponding fraction was freeze-dried prior to dry heating in an oven.
[b] Numbers in parentheses give the average net revertants/g dry beef when the Salmonella mutagen assays lacked S-9.
[c] Percent of the acid control lacking $NaNO_2$. Corresponding data for the standards IQ and Trp-P-2 were 95% and 0%, respectively.
[d] ND = not determined.

Supernatant 2 Heated at Higher Temperatures

The objective was to reach temperatures comparable to frying or broiling meat surfaces and yet avoid the use of high temperature boiling solvents and avoid extreme temperatures above 300°C in order to minimize mutagen formation via the pyrolysis of individual amino acids and proteins. Three heating methods were used: dry oven-baking (lyophilized material), aqueous solution (pressure), and frying (stainless steel griddle) within a ground beef matrix. Dry oven-baking was employed to simulate the dry heat conditions which likely prevail when sun-dried fish surfaces are broiled, when most of the H_2O has evaporated from the surface contact points of frying ground beef, or when dehydration occurs and scorched solids accumulate during the final stages of concentrating beef extracts to a thick dark paste (personal communication to R.T. Taylor from a commercial supplier). Dried fish and meat surface substances can then be subjected to much higher temperatures (150–300°C), since heat transfer is no longer consumed by the latent heat of vaporization of H_2O at these localized points. Figure 16 shows that no mutagenic activity appears after dry-heating S_2 for 3 hr at 100°C, but a large amount of activity forms at 200°C and much more at 300°C. At 300°C, TA 1538 activity increases almost linearly for the first 45 min, with no time lag as seen at 200°C. Table 6 summarizes the mutagenicity data for round steak fractions that were solution (pressure) or dry-heated. Again, low molecular weight compounds in the S_2 fraction are the sources of the activities which are two orders of magnitude greater (Table 6) than that of unstimulated 30 hr boiled S_2 (Table 5). Especially interesting are the acid-nitrite resistances. Whereas only 47% of the TA 1538 activity in boiled S_2 is nitrite-resistant (Table 5), about 60% is resistant in solution (pressure) heated S_2 and about 90% is resistant in oven-baked S_2 (Table 6).

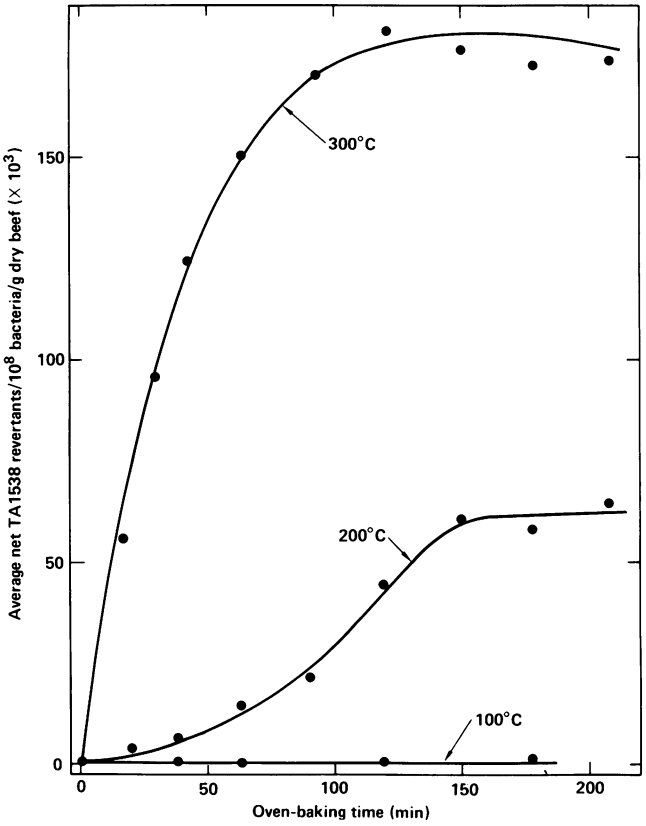

FIGURE 16. Effect of dry heating time at three temperatures on the TA 1538 activity of oven-baked, lyophilized supernatant 2.

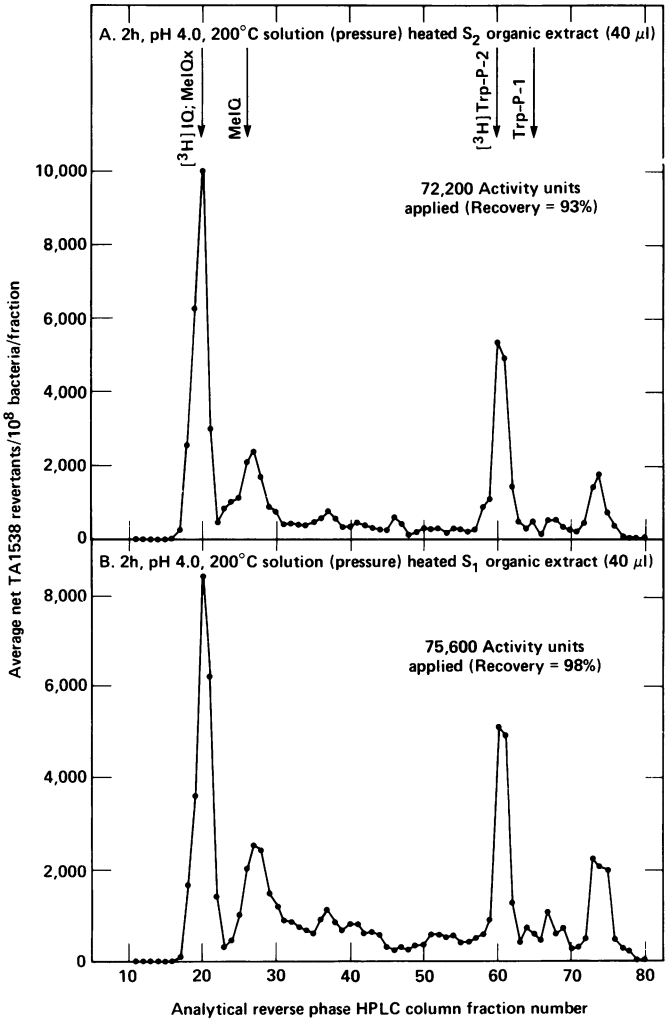

FIGURE 17. Analytical reverse phase HPLC of the alkaline organic extracts from 2 hr aqueously (Parr bomb) heated supernatant 2 (*A*) and supernatant 1 (*B*). In parts (*A*) and (*B*), 40 μL of 500 μL total extract sample were applied to the column.

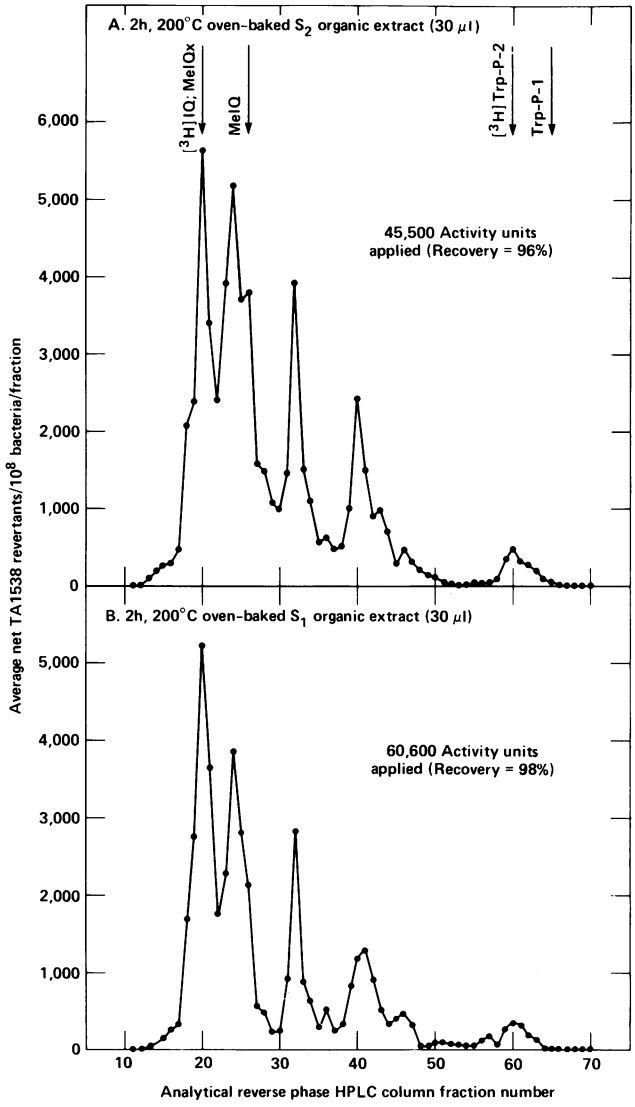

FIGURE 18. Analytical reverse phase HPLC of the alkaline organic extracts from 2 hr dry-heated supernatant 2 (*A*) and supernatant 1 (*B*). In parts (*A*) and (*B*), 30 μL of 1.3–1.7 mL total extract samples were applied to the column.

Comparison of the analytical reverse phase HPLC elution profiles for boiled S_2 (Fig. 7*A*), 200°C solution (pressure) heated S_2 (Fig. 17*A*), and 200°C dry-heated S_2 (Fig. 18*A*) confirms that the activities are shifted toward IQ-type elution patterns. At 200°C, they are also more complex with respect to the numbers of mutagens generated. However, 200°C solution (pressure) heated S_2 still contains a distinct major peak of activity that coelutes with [^{3}H]Trp-P-2 (Fig. 17*A*). This is consistent with 40% of the TA 1538 activity displaying non-IQ-type sensitivity to acid nitrite (Table 6). In contrast, this peak is a minor part of the activity profile for 200°C dry-heated S_2 that contains a higher proportion of more polar mutagenic activity (Fig. 18*A*). This agrees with only 10% of the 200°C dry heated S_2 activity being nitrite-sensitive or non-IQ-type mutagens (Table 6). The close similarities of the S_1 versus S_2 activity profiles within Figures 17 and 18 indicate that the 30 min boil needed to prepare S_2 from S_1 (Fig. 1) does not influence the types of mutagens that subsequently form at 200°C. No pH adjustments of the S_2 were made prior to any of the dry heating experiments.

We selected 10 min/side at 200°C as representative cooking conditions for studying the effects of round steak fractions on the mutagenicity of fried hamburger. Previously, Bjeldanes et al. found that the TA 1538 activity in the outer layers of ground beef fried at this temperature reaches a maximum after 6 min/side and remains constant up to 20 min/side (*30*). Figure 19 demonstrates that uniformly kneading either lyophilized S_2 or <500 MW S_2 into ground beef patties increases their

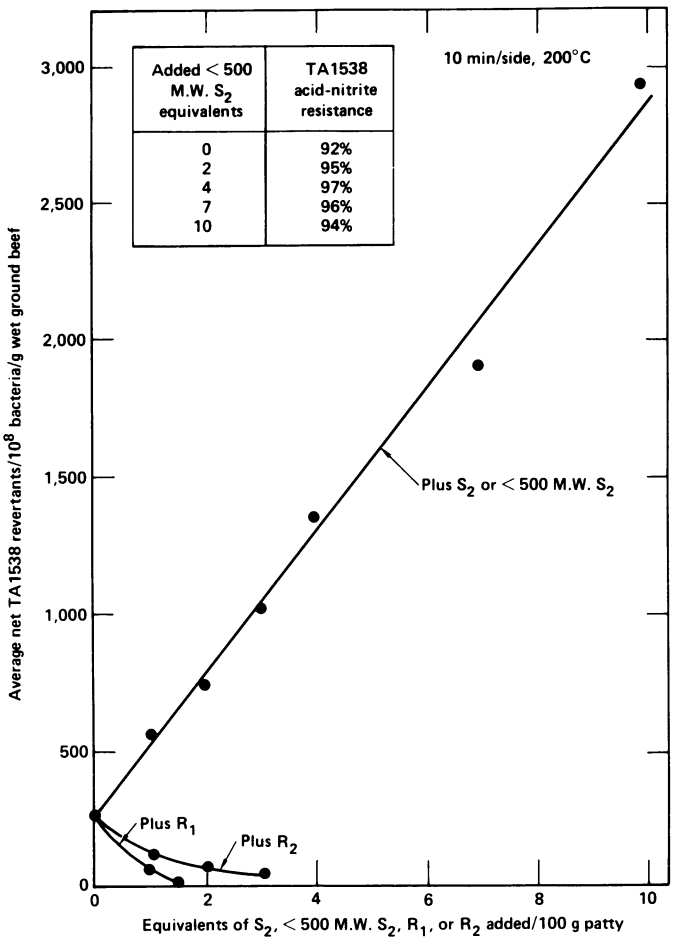

FIGURE 19. Effect of uniformly incorporated (kneaded) round steak fractions on the mutagenic activity of fried ground beef patties (100 g initial weight).

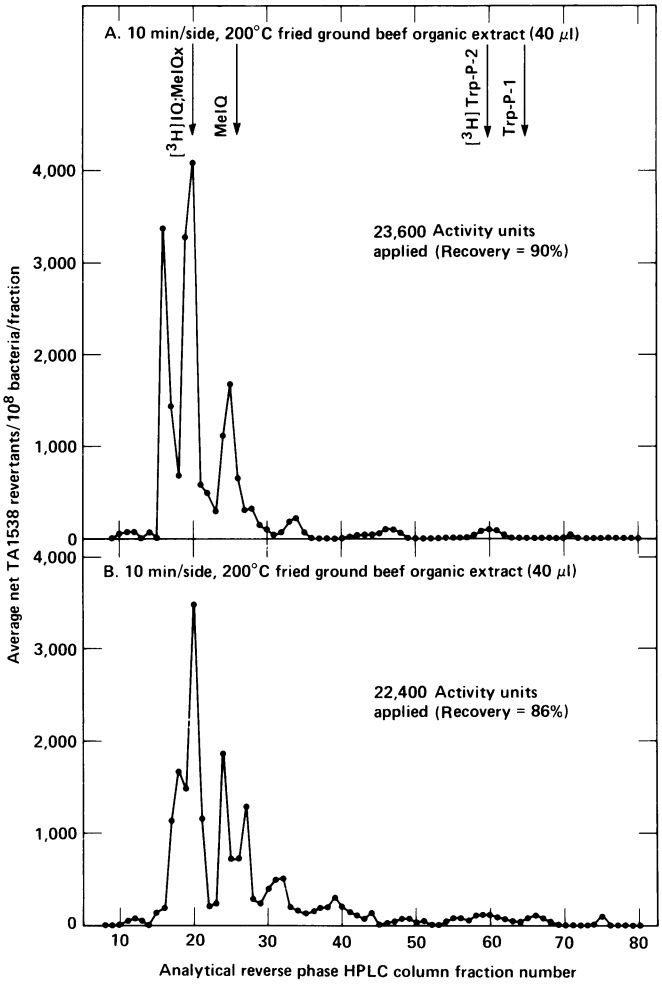

FIGURE 20. Analytical reverse phase HPLC of 10 min/side, 200°C fried ground beef from two separate cooks and extractions (*A*) and (*B*).

subsequent mutagenic activity (outer 3 mm layers extracted and assayed). The observed increases are directly proportional to the number of 100 g wet beef equivalents that were added. Importantly, each successively added equivalent of S_2 or <500 MW S_2 boosts the mutagenic activity by a multiple of the basal mutagenicity (260 TA 1538 revertants/10^8 bacteria/g of wet patty). Moist R_1 and R_2 lower the mutagenic activity of the fried patties, due to the replacement of the endogenous S_2 precursors by inert, non-precursor materials (Fig. 19). When 1.5 equivalents of R_1 are incorporated/100 g final wet weight, one is essentially frying an "R_1 patty" because this fraction comprises most of the solids in round steak (Fig. 1). The S_2 or <500 MW S_2-promoted activity increments are about 95% acid-nitrite resistant, like the control patty activity (Fig. 19, inset). This indicates the presence of predominantly IQ-type mutagenic activity in both the control and the stimulated fried hamburgers. Accordingly, despite some cook to cook variations, most of the control hamburger activity elutes from the analytical reverse phase HPLC column in fractions 13-35, i.e., in the same region as polar IQ-type mutagens (Fig. 20). The same applies for the mutagenic activity produced from the incorporated <500 MW S_2 substances (Fig. 21). In the less polar elution region of these profiles, there is definitely an enhancement of activity in fractions 55–65. However, it represents 10% or less of the total recovered activity.

Discussion

Several groups have examined the effects of time-temperature on the mutagenicity of fried ground beef or boiled-dehydrating beef stock (*23,30,39–41*). Bjeldanes et al. fitted their fried hamburger data to an equation in which the mutagenic activity has a very large exponential dependency on temperature at the ground beef surface-cooking surface interface (*30*). However, the mutagenicity of fried ground beef is also markedly dependent on the initial H_2O content of the patty. It increases 9-fold as the starting H_2O content is varied

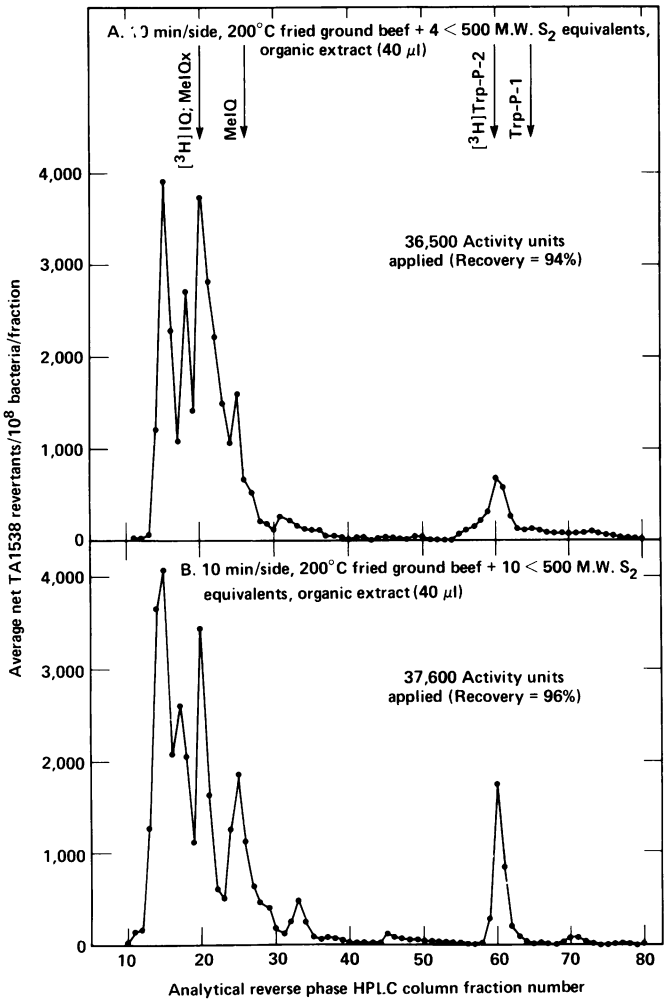

FIGURE 21. Analytical reverse phase HPLC of ground beef fried 10 min/side at 200°C with 4 added equivalents of <500 MW supernatant 2 (*A*) and 10 added equivalents of <500 MW supernatant 2 (*B*).

from 40% to 70% (*30*). Also, the heat transfer influences the rate of H_2O evaporation which in turn influences the temperature of the meat surface or beef extract paste at a given time point. These complications, coupled with an absence of any direct data on the sources of the mutagenic activity within frying ground beef or dehydrating beef paste, make the effects of H_2O difficult to assess. Since small soluble substances in S_2 appear to be the mutagen precursors, we used this beef fraction to examine the effects of both temperature and H_2O on mutagenic activity. Comparisons were made under conditions (constant volume boiling, solution [pressure] heating, and dry heating) in which H_2O evaporation was not an additional variable. Our results with S_2 confirm the overriding dependence of total activity on temperature (Tables 5 and 6). They also show that the number and types of mutagens change with temperature. In solution, many more mutagens form from S_2 after 2 hr at 200°C (Fig. 17*A*), than after 30 hr at 98 to 100°C (Fig. 7*A*). Yet, the proportion of non-IQ-type activity is still significant in 200°C solution (pressure) heated S_2 (Table 6; Fig. 17*A*). In a lyophilized state, many more mutagens are also produced by oven-baking S_2 for 2 hr at 300°C (HPLC activity profile not shown), than at 200°C (Fig. 18*A*). In Table 6, note that S_2 produced the same amount of total activity after 2 hr at 200°C, whether it was solution or dry heated. However, a differing distribution of mutagens and a much higher proportion of IQ-type compounds are present in 200°C dry versus solution-heated S_2 (Fig. 17*A*, Fig. 18*A*; Table 6). We conclude that H_2O is an important reactant that influences the types of mutagens formed at a given temperature.

To validate that food-related heterocyclic amines form when S_2 is dry-heated for 2 hr at 200°C, several mutagenic HPLC peaks are being purified by the steps in Table 4. From one active peak we have isolated IQ and determined that 18 ng are formed per g dry beef equivalent (R. T. Taylor and E. Fultz, unpublished results). This is 13 times the amount of IQ formed by boiling S_2 for 30 hr at pH 4.0 (Table 5), but it represents only 9% of the total activity, instead of the 50% in boiled S_2 (Fig. 7*A*). The amounts of IQ formed/g of heated S_2 fraction are 28 ng (30 hr boiled) and 360 ng (2 hr, 200°C dry-heated). Interestingly, these values bracket the IQ contents that were estimated for Difco beef extract, 20 to 40 ng/g (*26*) and 41 to 142 ng/g (*25*). Yoshida et al. recently isolated several micrograms of IQ after dry-heating 14 g of creatine and 11.5 g of Pro for 1 hr at 180°C (*42*). Finding that CP doubles the formation of IQ from boiled S_2 (Table 5, Fig. 7*B*) also indicates that CP or one of its degradation products is a precursor for IQ, most likely the NH_2-imidazole ring moiety. However, it remains to be seen whether the much larger amounts of IQ generated from 2 hr, 200°C dry-heated S_2 are derived by utilizing Pro as a coprecursor. It seems doubtful. Based on our amino acid data (*21*), there are only 2.1 mg of Pro/g of freeze-dried S_2.

In contrast to a consensus that IQ is a major mutagen in commercial beef extracts (*24–26*), the amounts of IQ in fried or broiled beef and the role of fat in cooked beef mutagen formation need some clarification. The contents of IQ reported by various investigators varies from none (*24*), to 0.02 (*27*), to ~0.6 (*8,43*), to 20.1 (*43*) ng/g of cooked or uncooked beef. Spingarn et al. found that raising the fat content of ground beef patties from 6.9% to 16.4% increased their mutagenic activity, after frying, about 5-fold. Additional fat lowered this enhanced activity (*44*). A similar pattern was observed by M. Knize and J. S. Felton (personal communication). Weisburger's laboratory also reported that frying 10.6% fat-ground beef yields 0.53 ng, while frying 27.5% fat-ground beef results in 20.1 ng of IQ per g of original meat (*43*). This 40-fold increase in IQ (*43*) is not compatible with their previous fried beef mutagenic activities and their stimulations by fat (*44*).

From the standpoint of modeling or considerations of

mutagen formation pathways, it is important to know whether or not lipids (fats) are a source of essential coprecursors. If not, how do they indirectly affect the mutagenicity of cooked beef? Currently, we favor the view that lipids do not appreciably contribute coreactants for mutagen formation—at least in cooked beef and pork. First, small H_2O-soluble compounds in S_2 appear to be the precursors for mutagen production, even when they are incorporated into a hamburger matrix and fried (Figs. 19–21). If endogenous fats supply coreactants in Figure 19, they would have to be in sufficient excess to accommodate 10-patty equivalents of the S_2 precursors. Otherwise, the stimulation of mutagenic activity would not be linear. Second, in the preparation of S_2 (Fig. 1) most of the chloroform–methanol (2:1)-extractable lipids (11%) (*20*) go into the insoluble R_1 and R_2 fractions as protein-bound material. R_1 and R_2 did not stimulate fried hamburger mutagenic activity (Fig. 19). Third, any floating fat globules or small pieces of congealed fat are removed at the S_1 stage of our preparations and are also removed before commercial beef stocks are boiled. Fourth, lean pork, containing only 2% fat, generated very high levels of mutagenic activity when it was pan-broiled (*45*). Fifth, when ground beef (15% fat) was defatted to 0% with petroleum ether and then fried as patties, they produced the same amount of TA 1538 activity as fried nondefatted, control patties (*30*). It is possible that limited percentages of fat augment hamburger mutagenicity several fold by increasing the heat transfer rate which determines the H_2O loss-rate. This could be offset with further additions of fat which would lower the S_2 precursor content per unit weight of uncooked patty.

It was first suggested by Commoner et al. (*23*) that the mutagens in commercial beef extracts and fried hamburger might arise via Maillard "browning reaction" pathways in which reducing sugars and amino compounds are the initial reactants (*46*). By refluxing any one of several amino acids with creatine or creatinine (Cre) and D-glucose (Glc) for 2 hr at 128 to 130°C in a 6:1 diethylene glycol–H_2O solvent system, Jägerstad et al. obtained high levels of frameshift mutagenic activity (*18,47*). In this solvent system, refluxing 0.33 M Cre + 0.33 M Gly + 0.17 M Glc yielded MeIQx as the major mutagen (90% of the activity) (*48*) and 7,8-DiMeIQx as a minor mutagen (10% of the activity) (*49*). After refluxing for 2 to 3 hr in a 5:1 diethylene glycol–H_2O solvent system, Matsushima (*50*) also isolated and identified MeIQx and 7,8-DiMeIQx from Cre + Gly + Glc. From the refluxed combinations Cre + Ala + Glc, Cre + Thr + Glc, and Cre + Ala + ribose, MeIQx and 4,8-DiMeIQx were identified (*50*). These findings have created much interest because MeIQx is a mutagen that is common to both commercial beef extracts and fried beef (*8,24–27*). A possible route to the formation of both IQ and MeIQx was suggested in which the Maillard products 2-methylpyridine and 2,5-dimethylpyrazine, respectively, are key intermediates (*47*). However, these compounds stimulated the mutagenic activity of refluxed Cre + amino acid + Glc mixtures only 2-fold at most (*18,47*). We observed no enhancement by 2,5-dimethylpyrazine of the mutagenic activity of S_2 that was boiled ± CP (Table 2).

Although it is attractive to envision Maillard products as intermediates in the formation of NH_2-imidazoquinoxaline mutagens (MeIQx, 7,8-DiMeIQx and 4,8-DiMeIQx), their role in the formation of other kinds of food-related heterocyclic amines is less appealing. In ethylene glycol–H_2O solvent systems containing Cre + amino acid + Glc, mutagen thermogenesis seems to be highly directed toward NH_2-imidazoquinoxaline compounds (*48–50*). Very little of other types of heterocyclic amine mutagens seem to be generated. However, both boiled (Table 5) and dry-heated S_2 (discussed above) and boiled-dehydrated beef extracts (*24–26*) contain many parts per million of IQ. And some IQ can be formed without a reducing sugar, from dry-heated Cre + Pro (*42*). Moreover, it is not obvious how Maillard amino acid-sugar products might be involved in the formation of the Trp-P mutagens from boiled S_2 mixtures (Table 5). D-Glucose actually lowers the synergistic mutagenicity of boiled S_2^* in which the major products are IQ, Trp-P-2, and Trp-P-1 (Table 5). The inability of reducing sugars to stimulate the mutagenic activity of boiled S_2 ± CP (*36*) (Table 2) indicates that either they are not involved in our S_2 fraction, or else they are not rate limiting in aqueous solutions. Our inability to detect MeIQx in both boiled S_2 ± CP and boiled S_2^* may reflect the need for temperatures above 100°C in order to form significant amounts of NH_2-imidazoquinoxaline mutagens. Jägerstad et al. stated that no mutagenic activity was produced by boiling Cre + amino acid + Glc in H_2O (*18*). The rationale for a diethylene glycol–H_2O solvent mixture was to permit reflux temperatures approximately 130°C and thereby accelerate mutagen thermogenesis (*18,47*). It will be of interest to ascertain whether our S_2 that is aqueous (pressure) or dry-heated at 200°C (Table 6) contains significant amounts of MeIQx. It is reported that meat muscle contains only 0.1 to 0.2% (wet wt) glucose + glycolytic intermediates (*51,52*). Therefore, it would be of interest to know whether and how much MeIQx would be generated in 2 to 3 hr at 130°C if its concentration in Cre + amino acid + Glc mixtures was lowered to the mM levels that occur in meats and in our S_2 fraction. Autoclaving our S_2 for 2 to 3 hr at 121°C yields very little mutagenic activity (57 TA 1538 revertants/10^8 bacteria/g dry beef). Thus, an aqueous heating temperature of 120 to 130°C is inadequate for rapid mutagen formation from the concentrations of endogenous precursors that approximate those in beef muscle.

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